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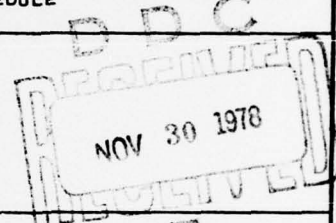
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EFFECTS OF LECTINS ON THE INTERACTION OF DIPHTHERIA TOXIN
WITH MAMMALIAN CELLS

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SUMMARY

The effects of concanavalin A (Con A) on diphtheria toxin-cell receptor interactions were studied. Con A had no measurable effect on toxin-receptor binding at 4°C but increased the total cell-associated toxin 4-5 fold at 37°C. An assay which distinguishes between cell surface bound and internalized toxin demonstrated that Con A reduces the percentage of internalized toxin while markedly increasing that found on the cell surface. These effects of Con A were blocked by α -methyl mannoside but not by colchicine. Succinyl Con A was as effective as Con A; other lectins, however, were less potent. It is concluded that Con A protects cells from diphtheria toxin by blocking its entry into the cell.

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Diphtheria toxin, the exotoxin produced by Corynebacterium diphtheriae, has been identified as the virulence factor in clinical diphtheria [1, 5, 15]. It is lethal for many animal species and cytotoxic for a large number of cultured mammalian cell lines. Diphtheria toxin is a 66,000 dalton protein which can be separated into two major fragments, designated A and B [1, 5, 15]. It is generally believed that fragment B mediates binding to the cell surface receptor, while fragment A catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide to a translocase essential for eukaryotic protein synthesis, elongation factor 2 (EF-2). ADP-ribosylated EF-2 is metabolically inactive and cytotoxicity therefore results from a toxin-induced inhibition of protein synthesis.

While the intracellular mechanism of action of diphtheria toxin has been elucidated, little is known of the molecular nature of the cell surface receptor or the means by which receptor-bound diphtheria toxin is internalized and processed by the cell. Draper et al. [2] have recently reported that certain lectins effectively protect Chinese hamster V79 cells from the action of diphtheria toxin. They hypothesized that protection results from a lectin-mediated block of toxin-receptor binding, indicating that the diphtheria toxin receptor contains N-acetylglucosamine or mannose elements.

We have recently developed a system which permits direct measurement of diphtheria toxin-receptor binding using a highly toxin-sensitive monkey kidney cell line [9, 11]. The effects of lectins in this system were studied and are reported here. Our data demonstrate that lectins do not block toxin-receptor binding but appear to prevent toxin internalization.

MATERIALS AND METHODS

Cells and cell culture

Seed stock for Vero and BS-C-1 cells was obtained from the American Type Culture Collection (ATCC), Rockville, Md. The cells were maintained in 75-cm² T-flasks (Costar #3075) with the medium and serum supplement recommended by ATCC. The Vero and BS-C-1 cell lines were used interchangeably since in previous work [9, 11] and in experiments presented here, they gave indistinguishable results.

Media and sera

All media, vitamins, antibiotics and amino acids were obtained from Grand Island Biological Company, Grand Island, N.Y. Fetal calf serum was purchased from Reheis Chemical Company, Phoenix, Ariz. The serum was heat-inactivated for 30 min at 56°C before use in cell culture.

Diphtheria toxin was obtained from Connaught Laboratories (Toronto) and purified by chromatography over DE-52 (Whatman). The final product was indistinguishable in cell culture experiments from purified diphtheria toxin (23 MLD/ μ g) supplied by Dr. A. M. Pappenheimer, Jr., Harvard University. Toxin concentration was determined using an extinction coefficient ($E_{1\text{ cm}}^{1\%}$) at 280 nm of 11.9.

Chemicals

Concanavalin A (Con A) was obtained from Pharmacia (Uppsala) and wheat germ agglutinin from Calbiochem (San Diego, Calif.). Succinyl Con A was prepared by the method of Gunther, et al. [6]. All other lectins were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Protein synthesis assay

Cells were grown in 24-well tissue culture plates (Costar #3424) to a concentration of $1-2 \times 10^5$ cells/well. On the day of experimentation,

the medium was replaced with Hanks' 199 supplemented with 10% heat-inactivated fetal calf serum and 25 mM Hepes, pH 7.4. Cells were incubated for 1 h at 37°C with the agents indicated, followed by challenge with toxin for various times. Tritiated leucine (New England Nuclear) was then added (1 μ Ci/well) and incubation continued for 1.5 h. The monolayers were then washed three times with Hanks' balanced salt solution and cells detached with 0.2 ml trypsin (trypsin-EDTA IX, Grand Island Biological Co.). After the addition of 0.8 ml of complete medium the entire contents of each well were transferred to a 12 x 75 mm test tube containing 1 ml of cold 10% trichloroacetic acid (TCA) and incubated overnight (4°C). The TCA-precipitable material was obtained by centrifugation, washed once in 5% TCA and once in methanol. The final pellets were solubilized in 0.5 ml NCS (New England Nuclear) and counted in Liquifluor-toluene (New England Nuclear) using a Searle Analytic Mark III liquid scintillation counting system.

Radiolabeling of toxin

Radiolabeled diphtheria toxin was prepared by the method of Roth [16] as previously described [9]; 60 μ g of toxin were labeled with 2 mCi of "Low pH" carrier-free sodium iodide-125 (New England Nuclear). The labeled toxin (25-40 μ l) was passed over a 3 ml Sephadex G-25 column equilibrated with 1 mg/ml bovine serum albumin in phosphate-buffered saline (PBS) and the excluded fraction pooled to a total volume of 1.0 ml. This material was 95% precipitable by cold 5% TCA and 90% precipitable using horse hyperimmune serum. Labeling usually proceeded to the level of $1-2 \times 10^7$ cpm/ μ g toxin (0.1-0.2 moles iodide per mole toxin). The labeling procedure had no detectable effect on

the biological activity of the toxin as measured by cytotoxicity assay [10].

Toxin-receptor binding assay

A detailed description of the binding assay has recently appeared [9]. Cells were grown in 24-well tissue culture plates as described above. On the day of experimentation, the growth medium was replaced with 1 ml of Hanks' 199 supplemented with 10% fetal calf serum and 25 mM Hepes, pH 7.4. ^{125}I -toxin or ^{125}I -toxin plus a 100-fold excess of unlabeled toxin was added to the wells (triplicate samples) and incubation carried out under the conditions indicated. The monolayers were then rinsed four times with Hanks' balanced salt solution, solubilized in 1.0 ml/well of 0.1 M NaOH and counted at 80% efficiency in a 1185 automatic gamma counting system (Searle Analytic Inc.). The level of specific binding was determined by subtracting counts obtained in the presence of excess unlabeled toxin from those obtained in the presence of ^{125}I -toxin alone. Standard errors were usually < 5%.

RESULTS

Effect of Con A on toxin-induced inhibition of protein synthesis

The effect of Con A on toxin-induced inhibition of protein synthesis in BS-C-1 cells is shown in fig. 1. Con A was found to protect cells from the action of diphtheria toxin as evidenced by a shift to the right of the dose-response curve. The magnitude of protection appeared similar to that reported by Draper et al. [2] with V79 cells although a direct comparison was not made. However, the maximal level of protection obtained with Con A was considerably less than that exhibited by two other protective agents, NH_4Cl [3, 8, 11] and adenosine-5'-tetraphosphate [12] (both at maximal protective concentrations). Since at high concentrations (about 100 $\mu\text{g/ml}$) Con A itself had a variable effect on protein synthesis (from 0-50% inhibition), the data are presented as percent of control, e.g., no toxin. Despite this complicating feature, protection was always observed.

Effects of Con A on toxin-receptor binding kinetics

The effects of Con A on the kinetics of diphtheria toxin-cell association are shown in fig. 2. As previously described [9], binding at 4°C follows a classical bimolecular kinetic pattern, reaching a plateau at 8-10 h. The addition of Con A had no effect on this pattern. At 37°C, binding kinetics are biphasic, normally increasing for 1-2 h after toxin addition and subsequently decreasing to a steady state, approximately 50% of the maximum. Con A markedly alters the 37°C kinetics, blocking the downward phase of the curve and eliciting a final binding level comparable to that achieved at equilibrium at 4°C.

The binding assay described here and routinely used in our laboratory measures total cell-associated radioactivity, a figure which

presumably represents both surface-bound and internalized diphtheria toxin. We have recently found that treatment of cells with a combination of pronase and inositol hexaphosphate (PIHP) releases surface-bound toxin into the medium, thus allowing differentiation between surface-associated and internalized toxin (Dorland, Leppla, and Middlebrook, submitted for publication). In cells exposed to toxin for up to 5 h at 4°C, PIHP continuously releases 85-90% of the total cell-associated radioactivity, indicating that essentially all of the toxin remains on the plasma membrane surface (fig 3A). An equivalent amount of cell-associated radioactivity was PIHP-releasable from cells incubated at 4°C in the presence of Con A (fig 3B). In contrast, at 37°C a substantial fraction of the cell-associated radioactivity became resistant to PIHP over time (fig 3C), presumably reflecting internalization of toxin. In the presence of Con A, however, nearly all of the cell-associated radioactivity remained PIHP-releasable (fig 3D), demonstrating that internalization was markedly inhibited. The same general pattern was observed in three separate experiments, which yielded % PIHP-releasable values of 88 ± 3 , 93 ± 1 , 34 ± 5 and 83 ± 4 (mean \pm S.E.) for 4°C, 4°C + Con A, 37°C, 37°C + Con A, respectively (all at 5 h).

Chemical and biophysical features of Con A effects on toxin-cell binding

Dose-response curves for the effects of Con A and its monovalent derivative, succinyl Con A, are shown in fig 4. Samples were harvested after a 5-h, 37°C incubation of cells with 125 I-diphtheria toxin, at which time the Con A effect appeared to be maximal (c.f. fig 2).

Thresholds for both the Con A- and the succinyl Con A-induced increase

in binding were seen at 1-5 $\mu\text{g/ml}$, while 100 $\mu\text{g/ml}$ were required to approach the 4°C binding level. Since the identical dose-response curves were unexpected, we carried out hemagglutination and mitogenicity tests to assure the authenticity of our succinyl Con A preparation; in both assays the results agreed well with literature values [6].

Many of the biological responses of cells to Con A are reversed by microtubule depolymerizing agents such as colchicine. The effect of colchicine in this system is shown in Table 1. Control experiments demonstrated that colchicine itself had no measurable effect on either the cytotoxic response to diphtheria toxin [12] or the binding kinetics of ^{125}I -diphtheria toxin at 4° or 37°C (data not shown). Clearly, when added with Con A, colchicine does not inhibit the lectin's effect on diphtheria toxin cell binding. Since this too was an unanticipated result, we tested the colchicine in a Con A-mitogenicity assay and found it had the expected inhibitory action [7].

Effects of other lectins on toxin-cell associates

Several other lectins have effects on diphtheria toxin cell association as is shown by the data in Table 2. Con A, added for reference purposes, brought about a large increase in binding after 5 h at 37°C. Addition of methyl mannoside blocked 80% of the Con A-induced increase while the glycoside alone had little or no effect. Wheat germ agglutinin and lentil lectin were about 70% and soybean lectin about 30% as effective as Con A. Ricins I and II and gorse lectin had no detectable effects on toxin-cell binding.

DISCUSSION

In this work we have studied the effects of lectins (principally Con A) on diphtheria toxin-receptor binding and inhibition of protein synthesis using highly sensitive mammalian cells [9, 11]. Draper et al. [2] previously demonstrated that lectins can block diphtheria toxin-induced inhibition of protein synthesis in hamster V79 cells. Our protein synthesis data with Vero and BS-C-1 cells are in good agreement with those observations. While acknowledging other possibilities, Draper et al. [2] suggested that lectins inhibit the action of diphtheria toxin by competing for the toxin receptor site. This in turn implies that the diphtheria toxin receptor may, in part, be a carbohydrate, specifically N-acetylglucosamine or mannose. The Vero or BS-C-1 cell system permits a direct measure of toxin-cell binding [9] and our data are incompatible with the above interpretation. Clearly, with Vero and BS-C-1 cells, Con A does not inhibit toxin-receptor binding. Indeed, at 37°C, Con A induces an apparent 4- to 5-fold increase in binding by 5 h (figs 2 and 3, and Table 1). At 4°C Con A has no measurable effect on binding up to a concentration of 100 μ g/ml. Therefore, although it is possible that mechanisms may vary from cell line to cell line, lectin protection from diphtheria toxin should not be taken as general evidence that its receptor contains a carbohydrate moiety.

Since the protective action of Con A cannot be attributed to a block of toxin binding, its effects on later stages of the intoxication process were investigated. One possibility is that Con A gains entrance to the cell cytoplasm and inhibits the toxin-catalyzed ribosylation of EF-2. We tested the effects of Con A on in vitro diphtheria

toxin-catalyzed ribosylation and found no inhibition up to a concentration of 100 $\mu\text{g/ml}$ (data not shown). Although this approach does not rule out the possibility that a fragment of Con A may act as an inhibitor, it appears unlikely that Con A affects the ribosylation stage of toxin action.

A much more likely explanation consistent with our data is that Con A blocks the internalization of diphtheria toxin. At 4°C, under conditions in which transport is minimal and the toxin is thought to remain exclusively on the outer surface of the cell membrane [1, 5, 9, 15], treatment with PIHP releases essentially all the cell-associated radioactivity into the medium. At 37°C, however, the fraction of PIHP-releasable radioactivity decreases with time, indicating a progressive internalization of surface-bound toxin; this decrease is blocked by Con A. We conclude, therefore, that the protective effect of Con A results from a lectin-mediated block of toxin internalization.

The ability of Con A to inhibit the mobility of membrane receptors has been reported by several laboratories [4]. Con A has been shown to inhibit patching and capping of various surface receptors on lymphocytes and numerous established cell lines [4, 14]. This inhibition is reversed by microtubule depolymerizing agents such as colchicine [7] and cannot be elicited by succinyl Con A [4, 6], a monovalent derivative of Con A. As assayed in our system, Con A and succinyl Con A are equally effective at altering the 37°C binding pattern. Moreover, neither compound's action is blocked by colchicine. This suggests that the ability of Con A to prevent diphtheria toxin internalization is not directly related to its

ability to cross-link membrane surface structures or to mobilize various colchicine-sensitive cytoskeletal components. Whatever the exact mechanism involved, lectin appear to be a valuable tool for studying how diphtheria toxin crosses the cell membrane.

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REFERENCES

1. Collier, R J, Bacteriol rev 39 (1975) 54.
2. Draper, R K, Chin, D & Simon, M I, Proc natl acad sci US 75 (1978) 261.
3. Duncan, J L & Groman, N B, J bacteriol 98 (1969) 963.
4. Edelman, G M, Science 192 (1976) 218.
5. Gill, D M, Pappenheimer, A M, Jr. & Uchida T, Fed proc 32 (1973) 1508.
6. Gunther, G R, Wang J L, Yahara, I, Cunningham, B A & Edelman, G M, Proc natl acad sci US 70 (1973) 1012.
7. Gunther, G R, Wang, J L & Edelman, G M, Exp cell res 98 (1976) 15.
8. Ivins, G, Saelinger, C B, Bonventre, P F, & Woscinski, C, Infect immun 11 (1975) 665.
9. Middlebrook, J L, Dorland R B & Leppla, S H, J biol chem 253 (1978) in press.
10. Middlebrook J L & Dorland R B, Can j microbiol 23 (1977) 175.
11. Middlebrook J L & Dorland R B, Can j microbiol 23 (1977) 183.
12. Middlebrook J L & Dorland R B, Infect immun 16 (1977) 232.
13. Middlebrook J L & Dorland R B, Abstracts of the Annual Meeting of the American Society for Microbiology 1977, American Society for Microbiology, Washington, D.C. p. 17.
14. Nicolson, G L, Int rev cytol 39 (1975) 89.
15. Pappenheimer, A M, Jr & Gill, D M, Science 182 (1973) 353.
16. Roth, J, Methods enzymol 37B (1975) 223.

Table 1. Effect of colchicine on Con A-induced increase of toxin-cell binding

Sample	Incubation time (h)	Specific cell-associated counts ^a
Control	1.5	4700 \pm 140
Control	5	2410 \pm 100
Con A (100 μ g/ml)	5	8240 \pm 700
Con A (100 μ g/ml) + colchicine (10^{-4} M)	5	8150 \pm 470

The agents to be tested were added to Vero cells followed by 125 I-toxin (0.03 μ g/ml) or 125 I-toxin plus unlabeled toxin (3 μ g/ml). After incubation at 37°C for the indicated times, specific cell-associated counts were assayed as in Materials and Methods.

^a Values are the means \pm S.E. (n = 3).

Table 2. Effect of lectins on diphtheria toxin-cell binding^a

Lectin added ^b	Specific cell-associated radioactivity
None	760 \pm 140
Con A	3400 \pm 300
Con A + α -methyl mannoside	1290 \pm 410
α -methyl mannoside	380 \pm 50
Wheat germ agglutinin	2520 \pm 520
Gorse lectin	780 \pm 140
Lentil lectin	2710 \pm 360
Soybean lectin	1560 \pm 380
Ricin I	510 \pm 70
Ricin II	740 \pm 20

^aVero cells in phosphate buffered saline-10% fetal calf serum were incubated with ¹²⁵I-toxin (0.03 μ g/ml) or ¹²⁵I-toxin plus unlabeled toxin (3 μ g/ml) in the presence of the indicated agent for 5 h at 37°C. Specific cell-associated radioactivity was measured as explained in Materials and Methods. Values are means \pm S.E. (n = 3).

^bAll lectins were used at a final concentration of 100 μ g/ml. α -Methyl mannoside was used at a final concentration of 50 mM.

FIGURE LEGENDS

Fig. 1. Abscissa: Toxin concentration ($\mu\text{g/ml}$); ordinate: % control protein synthesis. (\bigcirc), no agent; (\bullet), Con A, 100 $\mu\text{g/ml}$; (Δ), adenosine-5'-tetrphosphate, 0.5 mM; (\square), NH_4Cl , 0.5 mg/ml. Effect of Con A on diphtheria toxin-induced inhibition of protein synthesis. Vero cells were preincubated 1 h (37°C) with the indicated agent followed by a 5-h incubation with toxin. Protein synthesis was then measured as in Materials and Methods.

Fig. 2. Abscissa: time (hours); Ordinate: specific cell-associated radioactivity ($\text{cpm} \times 10^{-3}$). (\bullet), 4°C ; (\blacksquare), 4°C plus Con A; (\bigcirc), 37°C ; (\square), 37°C plus Con A. Effects of Con A on the kinetics of toxin-cell association. Vero cells were incubated with ^{125}I -toxin (0.03 $\mu\text{g/ml}$) or ^{125}I -toxin plus unlabeled toxin (3 $\mu\text{g/ml}$) at 4 or 37°C in the presence or absence of 100 $\mu\text{g/ml}$ Con A. At the indicated times samples were processed as in Materials and Methods to measure the specific cell-associated toxin.

Fig. 3. Abscissa: time (hours); Ordinate: specific radioactivity (cpm $\times 10^{-3}$). (○), supernatants; (Δ), cell pellets: A, 4°; B, 4° plus Con A; C, 37°; D, 37° plus Con A. Effect of Con A on toxin internalization. Vero cells were incubated with 125 I-toxin (0.03 μ g/ml) or 125 I-toxin plus unlabeled toxin (3 μ g/ml) at 4 or 37°C, with or without 100 μ g/ml Con A. At the indicated times, the monolayers were washed 3 times with 1 ml cold Hanks' balanced salt solution and 0.5 ml of 2.5 mg/ml pronase-10 mg/ml inositol hexaphosphate (cold) were added. After 1 h at 4°C, 0.5 ml fetal calf serum was added and the (detached) cells removed to be centrifuged at 7,000 g for 1 min. The supernatants were removed and counted separately from the cell pellets.

Fig. 4. Abscissa: lectin concentration (μ g/ml); Ordinate: specific cell-associated radioactivity (cpm $\times 10^{-3}$). (○), Con A; (□), succinyl Con A. Dose-response of Con A and succinyl Con A effects on toxin-cell association. Vero cells were incubated with 125 I-toxin (0.03 μ g/ml) or 125 I-toxin plus unlabeled toxin (3 μ g/ml) in the presence of the indicated concentration of Con A or succinyl Con A. After 5 h at 37°C samples were processed as in Materials and Methods to measure the specific cell-associated toxin.

